IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Art Unit: 1642
Fernando DOÑATE *et al.*	Examiner: David Blanchard
Serial. No. 10/074,225	
Filed: February 14, 2002	Atty. Docket No. 28932.0004
For: HISTIDINE PROLINE RICH GLYCOPROTEIN (HPRG) AS AN ANTIANGIOGENIC AND ANTI-TUMOR AGENT	Customer No. 30827

DECLARATION OF FERNANDO DOÑATE UNDER 37 CFR §1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Fernando Doñate, declare as follows:

1. I am a joint inventor of the subject matter of the pending claims and invented the subject matter of these claims in the United States prior to 05 February 2001.

2. The invention claimed in the above-identified application is directed to anti-angiogenic compounds (and methods) based on the protein histidine proline rich glycoprotein (HPRG).

3. I set forth my hypothesis that HPRG inhibits angiogenesis and the experimental results (inhibition of FGF-stimulated HUVEC proliferation and tubule formation) that proved this to be the case on pages 1, 6, 7 and 8 of Notebook 12 in the United States prior to 05 February 2001. Also stated prior to 05 February 2001is my conception that the H/P domain of HPRG was responsible for this activity, which was borne out by subsequent experiments. Evidence for the foregoing is found in Exhibit A, a copy of various pages from my laboratory notebook, with dates (obscured) at the bottom margin of each page, and in certain sections, to the right of the entries (obscured), all of which precede 05 February 2001.

4. I further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

4/18/05 Fernando Dñate

Date Fernando Doñate

1.1 Project Code No. HPRG

HPRG and angiogenesis 012

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[redacted]

Reasons to suspect that HPRG may possess anti- or pro-angiogenic properties:

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Because of similarities to HK:

- Both are abundant serum multi-domain proteins, containing cystatin-like domains and having a diverse array of activities.
- Sequence similarities between HK-D5 and His-Pro rich domain of HPRG.
- Both bind Zn+2/Cu+2/heparin/complement component C1q/thrombospondin.

Others:

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- HPRG inhibits endogenous and bFGF-stimulated 3T3 cells DNA synthesis.
- HPRG displaces bFGF from heparan sulfate proteoglycans, which may promote or inhibit bFGF mediated angiogenesis.
- Plasminogen-bound to cell–bound HPRG is more rapidly activated to plasmin by tPA. Plasmin, in turn, may activate several pro-MMPs promoting angiogenesis.
- HPRG can only bind to GAGs on the surface of ECS at acidic pH (hypoxia/ischemia/inflammation) or in the presence of Zn+2/Cu+2.
- HPRG also has diverse effects on T cells.

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HPRG (Histidine - Proline Rich Glycoprotein) is a very abundant protein in plasma, and, for the reasons stated above, it may have anti- or pro-angiogenic properties. The similarities (abundance of His residues) with domain 5 of Kininogen suggest that the His-Pro domain of HPRG may have anti-angiogenic properties.

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mando Dinate	[redacted]	Chen Wu	[redacted]

Project Code No. HPRG 012

TITLE HPRG and angiogenesis

From Page No. 6

RESULTS for HPRG: The experiments are done by Marian Plunkett and Scott Harris. Refer to their notebooks for details, methods etc:

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Cell Proliferation Assay with HUVECs + bFGF

Compound Tx			Compound Tx bFGF HUVEC-Medium/Control HUVEC-Medium EBM-10% FBS Gibco			EBM-2%Clonetechs		
SFM		1.03	Control	10%	2.75	Control	Control	1.06
SFM		0.84	Control	5%	2.90	Control	Control	1.12
FBS 10%		2.20	Control	2.50%	1.55	Control	Control	0.86
FBS 5%		2.63	Control	1.25%	1.69	Control	Control	1.00
FBS 2.5%		2.51	bFGF 2ng/	10%	3.63	bFGF	2 ng/mL	1.71
HGPR	12 µM	1.11	bFGF 2ng/	5%	3.32	bFGF	2 ng/mL	1.79
HGPR	6 µM	1.06	bFGF 2ng/	2.50%	2.83	bFGF	2 ng/mL	1.74
HGPR	3 µM	1.08	bFGF 2ng/	1.25%	2.21	bFGF	2 ng/mL	1.49
bFGF 2 ng/mL		1.67						

3000 cells/well
Cultured 2 days EBM-10%FBS complete growth medium
Cultured O/N EBM-2%FBS
Control cells: EBM-2%FBS
Growth factor control cells: EBM-2%FBS +2 ng/mL bFGF
Compound Tx cells: EBM-2%FBS + 2 ng/mL bFGF

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Medium Control 0.4

[redacted]

Control AVG	1.033467
Stdev	0.116975
bFGF AVG	1.214642
Stdev	0.101502
bFGF-MC/Control-MC	1.286006

	2ug/mL	stdev	0.4mg/mL	stdev
ATN154	1.258567	0.136817	1.247333	0.045025

	3uM	+20uM zn
HPRG	1.025367	1.037733
stdev	0.041436	0.03571

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	2% serum	5% serum	10% serum
	1.723513	1.578125	1.344963
+2ng/mL bFGF	1.890667	1.830263	1.498525

[redacted]

ATN154 2ug/mL	1.355346
0.4ug/mL	1.337613
HPRG 3uM	0.987213
+20uM Zn	1.006735
2% serum	2.089317
+bFGF	2.353189
5% serum	1.859806
+bFGF	2.257834
10% serum	1.491732
+bFGF	1.734148

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Recorded by: F. Donato Date [redacted] Verified by: Chen Wang Date [redacted]

8 012 TITLE HPRG and angiogenesis

From Page No. 3

RESULTS:

- Cell proliferation Assay using HUVECS +/- bFGF*:

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[HPRG]	% of inhibition of bFGF stimulation of HUVECS: No Zn^{+2}	% of inhibition of bFGF stimulation of HUVECS: $+Zn^{+2}$	No Zn^{+2} OD	$+Zn^{+2}$ OD
3 µM	61.29	61.29	1.24	1.24
1.5 µM	16.13	30.65	1.52	1.43
0.5 µM	-4.84	-19.35	1.65	1.74
100 nM	-8.06	-9.68	1.67	1.68
1 nM	-70.97	-75.81	2.06	2.09
bFGF			[redacted] 1.62	

[redacted]

µM HPRG			% inhibiton	+ 10 µM Zn % inhibition
3	0.71	0.98	180.56	105.56
1.5	0.95	0.95	113.89	113.89
0.5	1.29	1.29	19.44	19.44
0.1	1.18	1.17	50.00	52.78
0.05	1.34	1.36	5.56	0.00
bFGF	1.36			

- Tubule formation assay*:
  HPRG inhibited tubule formation by HUVECS in (250nM) matrigel, and matrigel supplemented with bFGF, or VEGF. The experiment is done twice

- HPRG @ 1µM is not ~~cytotoxic~~ cytotoxic to HUVECS*

*: All these assays are done by Marion and Scott. Data of them can be found in their notebooks

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HPRG and angiogenesis

No. 8

CONCLUSIONS and HYPOTHETICAL MODE OF ACTION

HPRG FD seems to have anti-angiogenic properties based on the inhibition of HUVECs upon bFGF stimulation, and inhibition of tubule formation by HUVECs in Matrigel alone, or Matrigel supplemented with bFGF or VEGF. Furthermore, HPRG is not cytotoxic to HUVECS as determined by counting control cells and cells incubated with 1 μM HPRG. All those experiments were performed by M. Plunkett and S. Harris and recorded in their notebooks. It is unknown yet whether the entire protein is needed for this anti-angiogenic activity or only one or mode of the domains. It is possible, based on homology to D5 of HK, that the His-Pro domain of HPRG is needed for this activity. Thus, following is a hypothetical mechanism for the anti-angiogenic activity of this protein. HPRG circulates in plasma at high concentration (1.5 μM). At regions of hypoxia (pro-angiogenic environments) or possibly, ischemia, the local pH may have dropped so that the abundant His of the His-Pro domain became fully protonated. Alternatively or simultaneously, the local concentration of metal such as Cu+2 or Zn+2 may increase and the metal binding sites on HPRG His-Pro domain became saturated. Either situation facilitates binding of HPRG to heparan sulfate like structures on the surface of endothelial cells (ECs). The local environment modifies the protein so that it can now bind to the surface of ECs. Plasminogen bound to cell-bound HPRG becomes activated more rapidly by tPA than free plasminogen. Plasminogen, which is abundant in these settings, is bound to HPRG and gets converted into plasmin while bound to ECs. In turn, plasmin cuts within the different domains of HPRG eventually liberating the His-Pro domain. This domain can now exert its anti-angiogenic properties. A possible therapy for pathological processes depending on angiogenesis (cancer, rheumatoid arthritis...) may be articulated by administration of HPRG or domains of each, or peptides based on it, or other molecules based on properties of HPRG.

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HPRG may also have pro-angiogenic properties due to the fact that 50% of plasminogen circulates bound to HPRG. Plasminogen bound to cell-bound HPRG becomes activated more rapidly by tPA than free plasminogen. The pro or anti-angiogenic properties of HPRG may be exerted depending on the local environment. It may also be possible that different domains may be responsible for these opposed properties.

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[CANCER RESEARCH 64, 5812–5817, August 15, 2004]

Peptides Derived from the Histidine-Proline Domain of the Histidine-Proline-Rich Glycoprotein Bind to Tropomyosin and Have Antiangiogenic and Antitumor Activities

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ABSTRACT

The antiangiogenic activity of the multidomain plasma protein histidine-proline-rich glycoprotein (HPRG) is localized to its histidine-proline–rich (H/P) domain and has recently been shown to be mediated, at least partially, through binding to cell-surface tropomyosin in fibroblast growth factor-2-activated endothelial cells (X. Guan *et al.*, Thromb Haemost, in press). HPRG and its H/P domain, but not the other domains of HPRG, bind specifically and with high affinity to tropomyosin. In this study, we characterize the interaction of the H/P domain with tropomyosin and delineate the region within the H/P domain responsible for that interaction. The H/P domain of HPRG consists mostly of repetitions of the consensus sequence [H/P][H/P]PHG. Applying an *in vitro* tropomyosin binding assay, we demonstrate that the synthetic peptide HHPHG binds to tropomyosin *in vitro* and inhibits angiogenesis and tumor growth *in vivo*. The affinity for tropomyosin increases exponentially upon multimerization of the HHPHG sequence, with a concurrent increase in antiangiogenic activity. Specifically, the tetramer $(HHPHG)_4$ has significant antiangiogenic activity in the Matrigel plug model (IC_{50} ~600 nM) and antitumor effects in two syngeneic mouse tumor models. Thus, we show that a 16-mer peptide analogue mimics the antiangiogenic activity of intact HPRG and is also able to inhibit tumor growth, suggesting that cell surface tropomyosin may represent a novel antiangiogenic target for the treatment of cancer.

INTRODUCTION

We have described the antiangiogenic properties of the abundant plasma protein histidine-proline-rich glycoprotein (HPRG) and localized these properties to the His-Pro-rich (H/P) domain (1), a result that has recently been confirmed (2). In this regard, HPRG is functionally similar to an evolutionarily related protein, activated high molecular weight kininogen (HKa), which has also been demonstrated to inhibit angiogenesis (3–5). Rabbit and human HPRG are highly homologous and demonstrate similar biological activity (1, 6, 7). Thus, we have used rabbit HPRG (rbHPRG) for our studies rather than the human protein because it is more straightforward to isolate intact individual domains from rbHPRG (6, 7). Tropomyosin, which forms part of the actin cytoskeleton (8), has been identified on the surface of fibroblast growth factor-2 (FGF-2)-activated human umbilical vascular endothelial cells (HUVEC; Refs. 5, 9) and mediates the anti-angiogenic activity of HKa (5). The interaction of HPRG with endothelial cells has recently been described previously (9). rbHPRG binds with high affinity to HUVEC through its H/P domain and has at least two binding sites that exist on the surface of HUVEC: tropomyosin (high affinity) and heparan sulfate proteoglycans (moderate affinity). The interaction of HPRG with heparan sulfate proteoglycans has been studied in several model systems (10–12). As in the case of HKa, the antiangiogenic activity of the H/P domain of rbHPRG is mediated, at least partially, through tropomyosin located on the surface of endothelial cells. The interaction of rbHPRG and its H/P domain with tropomyosin requires Zn^{2+} or His protonation, which could occur under mildly acidic conditions such as those observed within a hypoxic region of a tumor (9).

The H/P domains of HPRG across different species are comprised primarily of repeats of conserved pentapeptides containing His, Pro, and Gly residues (6, 7, 13). A hybrid consensus sequence of human and rabbit HPRG that fully describes all possible permutations of the H/P domain in both species is designated [H/P][H/P]PHG (7). The rabbit H/P domain is comprised of two repeats of HHPHG, seven repeats of PPPHG, six repeats of HPPHG, and the sequence GFH-DHGPCDP PSHK at the COOH-terminus of the domain (7). The H/P domain of human HPRG contains other types of residues intercalated within 10 tandem repeats of the sequence HHPHG (7). Because the H/P domain of rabbit HPRG contains all of the determinants required for antiangiogenic activity (1), we inferred that the intervening sequences in human H/P are not necessary for activity and hypothesized that the binding to endothelial cell surface tropomyosin may occur through the repeats of pentapeptides containing the consensus sequence [H/P][H/P]PHG.

In this study, we define the basis for the interaction of the H/P domain of HPRG with tropomyosin and delineate the region within the H/P domain responsible for its interaction with tropomyosin and its antiangiogenic activity. We show here that the peptide HHPHG has the highest affinity toward tropomyosin as well as the most potent antiangiogenic activity of the 5-mer peptides tested and that the His residues are essential for both binding to tropomyosin as well as for the inhibition of angiogenesis. Furthermore, we demonstrate that multimers of the HHPHG sequence have increased antiangiogenic activity and affinity for tropomyosin with progressively increasing multimer size. Finally, the largest multimer tested, the tetramer $(HHPHG)_4$, also demonstrates substantial antitumor activity in two syngeneic [Lewis lung carcinoma (3LL) and B16F1] models of tumor growth in mice.

MATERIALS AND METHODS

Proteins, Peptides, and Materials. FGF-2 and vascular endothelial growth factor were purchased from Research Diagnostics, Inc. (Flanders, NJ). HKa was obtained from Enzyme Research Laboratories (South Bend, IN). Heparin was acquired from Sigma (St. Louis, MO). Intact protein and domains of rabbit HPRG were obtained as described previously (1). Peptides were synthesized and purified by Peptisyntha (Torrance, CA). HKa and HPRG were biotinylated using EZ-Link Sulfo-NHS-LC-biotin (Pierce, Rockford, IL) following the manufacturer's instructions.

Tropomyosin Competition Binding Assay. Chicken gizzard tropomyosin (Sigma) was dissolved in TBS [50 mM Tris, 140 mM NaCl (pH 7.5)]. Two hundred ng of chicken gizzard tropomyosin were added/well of a 96-well high-binding assay plate and incubated for 2 h at room temperature. The plate was washed with TBS-T [TBS with 0.05% Tween 80 (v/v)] and blocked with

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2% fish gelatin (Sigma). Biotin-HKa (10 nM) in TBS containing 10 μM $ZnCl_2$ was added to the plate together with the peptides. The plates were incubated at room temperature for 2 h and extensively washed with TBS-T buffer. Bound biotin-HKa was detected using avidin-horseradish peroxidase and the chromogenic substrate 3,3'-5,5'-tetramethylbenzidine (Roche, Indianapolis, IN). The indicated IC_{50} values were calculated by nonlinear fitting of experimental data using Prism.

Matrigel Plug Model. Cold Matrigel (500 μl) was mixed with heparin (50 μg/ml), FGF-2 (400 ng/ml), and the peptides to be tested. Positive control plugs did not contain the test peptides, and negative controls plugs did not contain the pro-angiogenic FGF-2. In some experiments, 800 ng/ml FGF-2, 300 ng/ml vascular endothelial growth factor and heparin were used. The Matrigel mixture was injected s.c. into 4–8-week-old female BALB/c nude mice, and the animals were sacrificed and the plugs recovered 5 days after injection. The plugs were then minced and homogenized with a tissue homogenizer, and hemoglobin levels in the plugs determined using Drabkin's solution according to the manufacturer's instructions (Sigma). Alternatively, the Matrigel plug vascularization was determined after i.v. injection of 100 μl of FITC-dextran (M_r 250,000; Sigma) into the tail vein followed by fluorimetric analysis of the plug after removal. Both methods of detection yielded similar results.

Modified Matrigel Plug Model. MatLyLu rat prostate cancer cells (2×10^6 cells) were mixed with Matrigel and the peptide to be tested and then injected into the flank of a mouse following the protocol described for the Matrigel Plug model. After 7 days, the animals were euthanized and the plugs removed and weighed. The plugs were then minced and homogenized with a tissue homogenizer, and hemoglobin levels were determined using Drabkin's solution according to the manufacturer's instructions.

Endothelial Cell Binding Assay. To assess protein binding, 30,000 HUVEC were plated in a 24-well plate and incubated overnight in M200 containing 2% serum. Medium was then changed to basal medium (M200) containing 2% fetal bovine serum (FBS) with or without 10 ng/ml FGF-2 and incubated for 4 h at 37°C. The medium was then aspirated, the plate placed at 4°C, and the desired amounts of biotin-rbHPRG and the peptide to be tested were added in 200 μl of HBS (pH 7.5) and 10 μM ZnCl. Nonspecific binding was determined by measuring binding in the absence of Zn^{2+}.

Endothelial Cell Proliferation Assay. HUVEC were cultured overnight in M200 containing 2% FBS. The following day, 3000 cells were plated in each well of a gelatin-coated 96-well plate. The cells were allowed to adhere and spread for 4–6 h, at which time, the medium was replaced with fresh medium containing 2% FBS, 1 ng/ml FGF-2, and varying concentrations of specific test compounds in the presence and absence of 10 μM $ZnCl_2$. Cells were then cultured for an additional 48 h, and relative cell numbers in each well were determined using the Cell Titer Aqueous Cell Proliferation Assay (Promega, Madison, WI).

Tumor Growth Inhibition Experiment and Cell Culture. The 3LL cells were a gift from the laboratory of Dr. Judah Folkman (Children's Hospital, Harvard Medical School). The cells were grown in DMEM, 10% FBS containing, 80 μM L-glutamine, and 0.15% sodium bicarbonate at 37°C in a humidified 5% CO_2 incubator. B16F1 cells were obtained from the laboratory of Dr. Ronald Goldfarb (University of North Texas Health Science Center) and were maintained in RPMI 1640 containing 10% FBS and 1% MEM nonessential amino acids at 37°C in a humidified 5% CO_2 incubator. For the tumor growth inhibition experiments, C57Bl/6 female mice were inoculated s.c. with 2.5×10^5 3LL cells or 7.5×10^4 B16F1 cells. After 4 days, peptide (dissolved in PBS) treatment was initiated and continued daily (Monday through Friday). Vehicle alone was used as a negative control, and metronomically dosed (170 mg/kg, once/week) cyclophosphamide (Sigma) was used as a positive control (14).

Statistical Analysis. Results were analyzed using the Student's *t* test. Data are presented as mean ± SD or mean ± SE (Fig. 7).

RESULTS

Identification of the Pentapeptide from the H/P Domain of HPRG with the Highest Affinity for Tropomyosin. To identify the peptide from the consensus sequence [H/P][H/P]PHG required for the binding of HPRG to tropomyosin, three peptides, PPPHG, HPPHG, and HHPHG, having free or capped termini (acetylated and amidated at the COOH- and NH_2-termini, respectively) were synthesized. We have previously demonstrated that the binding of HPRG and HKa to immobilized tropomyosin in a 96-well solid phase assay correlates with the binding to FGF-2-stimulated HUVECs (5, 9). HKa and HPRG could be used interchangeably in this assay, but HKa provides a superior signal-to-noise ratio and was thus used in all subsequent experiments. This assay was used to study the interaction of the H/P-derived peptides with tropomyosin. The six peptides (three capped, three uncapped) were screened for their ability to compete with biotin-HKa for binding to tropomyosin at concentrations of 50 and 200 μM (Fig. 1*A*). Only the uncapped forms of peptides HHPHG and HPPHG substantially inhibited binding in this assay, and all subsequent studies were carried out using these two peptides. The difference in affinity of HHPHG as a capped or uncapped peptide is shown as a dose titration curve in Fig. 1*B*. The IC_{50} for the uncapped peptide is ~150 μM, whereas the capped peptide competes with the binding of biotin-HKa with an IC_{50} ~1 mM, indicating that a free NH_2- and/or COOH-terminus are important for the interaction with immobilized tropomyosin. Similar results were found for capped and uncapped HPPHG (data not shown).

Ala Scanning Replacement of HHPHG and HPPHG. Ala scanning replacement of both peptides was carried out with the objective of assessing the relative contribution of each residue to tropomyosin

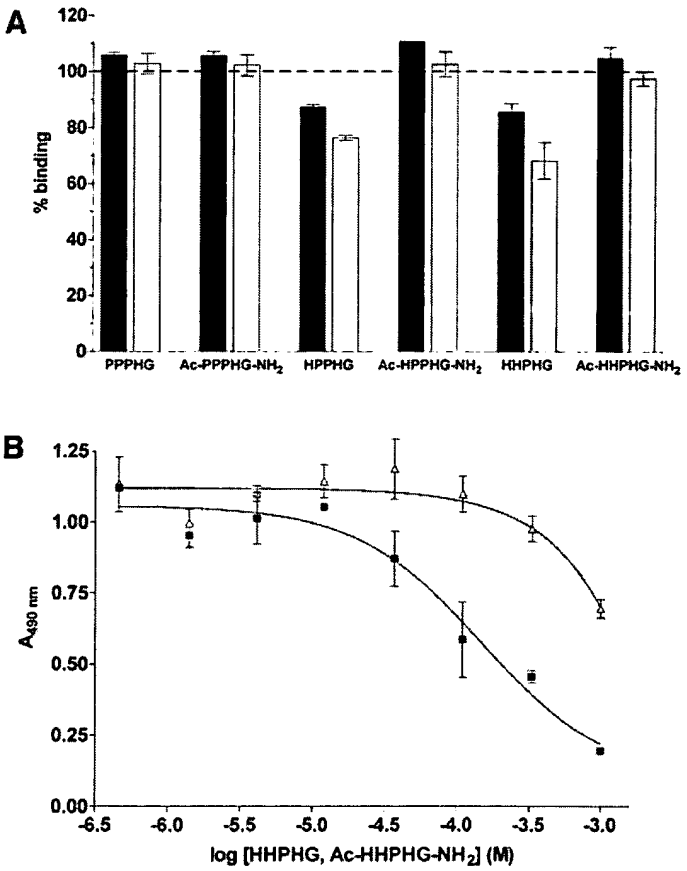

Fig. 1. Peptides from the consensus sequence of the H/P domain inhibit binding of biotinylated HKa to tropomyosin *in vitro*. *A*, 10 nM biotin-HKa, together with the peptide to be tested at 50 (■) or 200 (□) μM, were added in TBS [10 μM $ZnCl_2$ (pH 7.4)] to a 96-well plate previously coated with 200 ng of chicken gizzard tropomyosin. After incubation at room temperature and extensive washing, the bound protein was detected using avidin-horseradish peroxidase and a chromogenic substrate. The absorbance in the absence of peptide is considered no inhibition. *B*, 10 nM biotin-HKa was mixed with increasing concentrations of HHPHG (■) or Ac-HHPHG-NH_2 (△) as described for *A*. An IC_{50} was calculated by nonlinear fitting of the data.

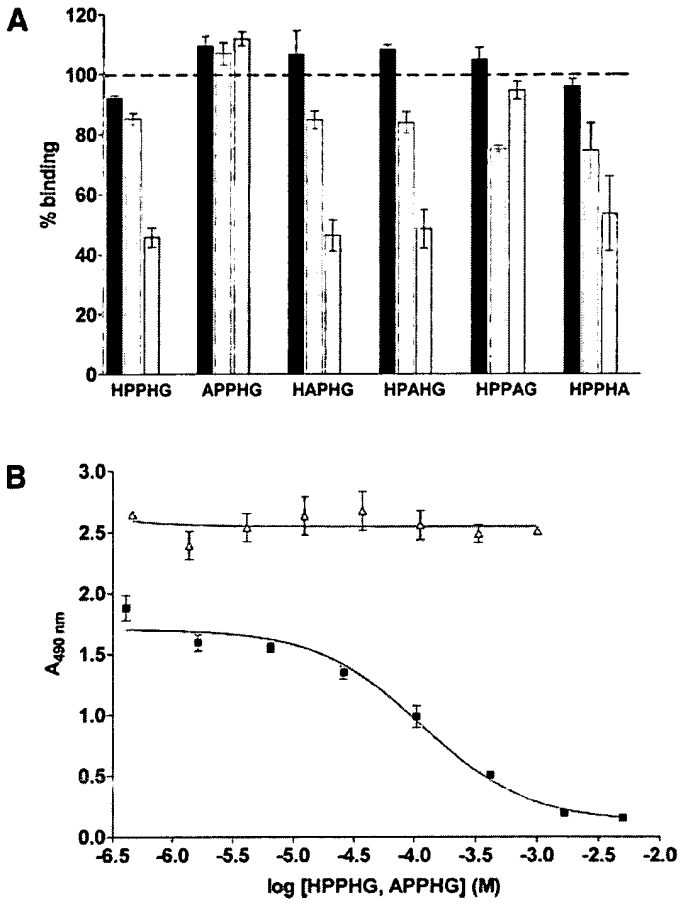

Fig. 2. Ala scanning replacement of HPPHG. *A*, peptides containing Ala replacement in each position of the peptide HPPHG were tested in the tropomyosin binding assay as described in Fig. 1*A*, at 10 (■), 100 (□) or 1000 (□) μM. The absorbance in the absence of peptide is considered no inhibition. *B*, 10 nM biotin-HKa are mixed with increasing concentrations of the HPPHG (■) or the APPHG (△) peptides as described in Fig. 1*B*.

binding. Replacement of either His residue in HPPHG with Ala significantly decreased the ability of the peptide to compete for binding to tropomyosin (Fig. 2*A*) and substituting the NH_2-terminal histidine completely abrogated the ability of the peptide to compete for binding to tropomyosin (Fig. 2*B*). A more detailed Ala replacement study was carried out with the HHPHG peptide (Fig. 3). The IC_{50} values of the variant peptides in the tropomyosin binding assay were normalized to the activity of HHPHG, IC_{50} = 155 ± 49 μM. As observed with the HPPHG peptide, the first His residue provides the largest contribution to binding. Substitution of the other His residues also decreased binding, but replacement at the non-His positions had no significant effect (Fig. 3). Truncation of the last Gly residue (HHPH) and even combining the truncation with the Ala substitution of the Pro (HHAH) had only a small effect on the binding affinity.

These results indicate that the activity of the peptides in the tropomyosin binding assay is His-residue dependent. His residues also bind to divalent cations (15), and thus, a trivial explanation for the inhibition of biotin-HKa binding to tropomyosin is that the peptides could simply be stripping away the Zn^{2+} required for this binding. To assess this possibility, we preincubated the peptides at three different concentrations close to their IC_{50} values with the same concentration of $ZnCl_2$ used in the assay (data not shown). If the peptides were prebound with Zn^{2+}, their capacity to inhibit biotin-HKa binding by competing for Zn^{2+} should be diminished. However, even after preincubation with Zn^{2+}, the peptides retained full inhibitory capacity, indicating that the inhibition of binding of biotin-HKa to tropomyosin by the peptides was not due to stripping Zn^{2+} from the assay. Because the affinity of the HHPHG peptide (IC_{50} = 154 ± 49 μM) is somewhat higher than that of the HPPHG peptide (IC_{50} = 242 ± 144 μM) in the tropomyosin binding assay, the HHPHG peptide was chosen for additional studies.

To test whether binding to tropomyosin *in vitro* correlated with antiangiogenic activity *in vivo*, pairs of peptides with low and high IC_{50} values in the tropomyosin binding assay were compared in both the Matrigel plug model and the modified Matrigel plug model in which the angiogenic stimulus is provided by tumor cells that are mixed with the Matrigel (1). The HHPHG peptide had significant inhibitory activity in both assays (Fig. 4), suggesting activity against both FGF-2 and tumor-driven angiogenesis. In contrast, the activity of the AHPHG peptide, which had the lowest affinity (highest IC_{50}) of all of the Ala mutants of the HHPHG series for tropomyosin, was significantly lower than that of HHPHG in both *in vivo* models (Fig. 4). Similar results were obtained with HPPHG (low IC_{50}, high antiangiogenic activity) and APPHG (high IC_{50}, low antiangiogenic activity; data not shown). These results demonstrate the correlation between tropomyosin binding *in vitro* and antiangiogenic activity *in vivo*. Furthermore, the fact that HHPHG and HPPHG have antiangiogenic activity is consistent with our hypothesis that the antiangiogenic activity of the H/P domain resides in the consensus sequence [H/P][H/P]PHG.

Tropomyosin Binding Affinity and Antiangiogenic Activity of Multimers of HHPHG. Although truncation of the last Gly residue in the HPPHG peptide and replacement of Pro residues with Ala had only minor effects on binding, structural roles for these two residues might not be observed when they are part of a small peptide but could become important if that sequence was contained in a larger peptide or protein. The H/P domain is known to adopt a poly-Pro type II helix (7). Thus, Gly residues may act as spacer residues, and Pro residues may promote the poly-Pro secondary structure in larger peptides. Therefore, these two residues were retained in each HHPHG subunit in our investigation of the effect of multimerization on binding and antiangiogenic activity. Capped and uncapped multimers (two to four repeats) of the HHPHG peptide were synthesized and tested in the tropomyosin binding and Matrigel plug assays (Table 1). A direct correlation between the number of repeats of the consensus sequence and the affinity for tropomyosin and antiangiogenic activity was

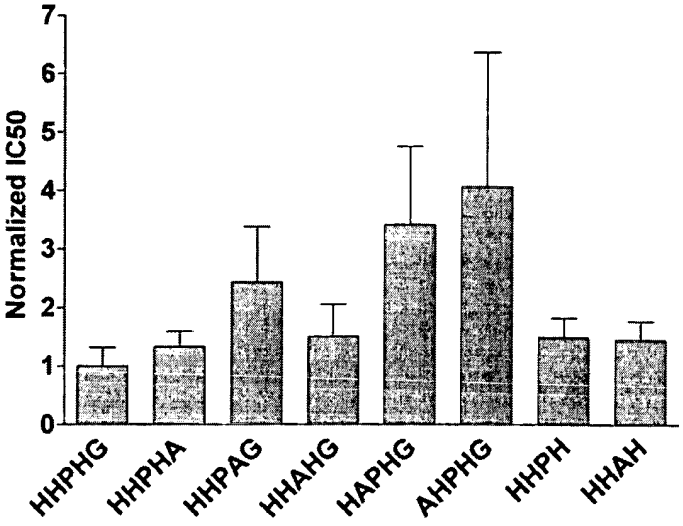

Fig. 3. Alanine scanning replacement of HHPHG. Peptides containing Ala replacement in each position of the peptide HHPHG were tested in the tropomyosin binding assay as described in Fig. 1. The data are the mean and SD of n = 4 experiments. The calculated IC_{50} values were normalized to the activity of HHPHG, IC_{50} = 155 ± 49 μM.

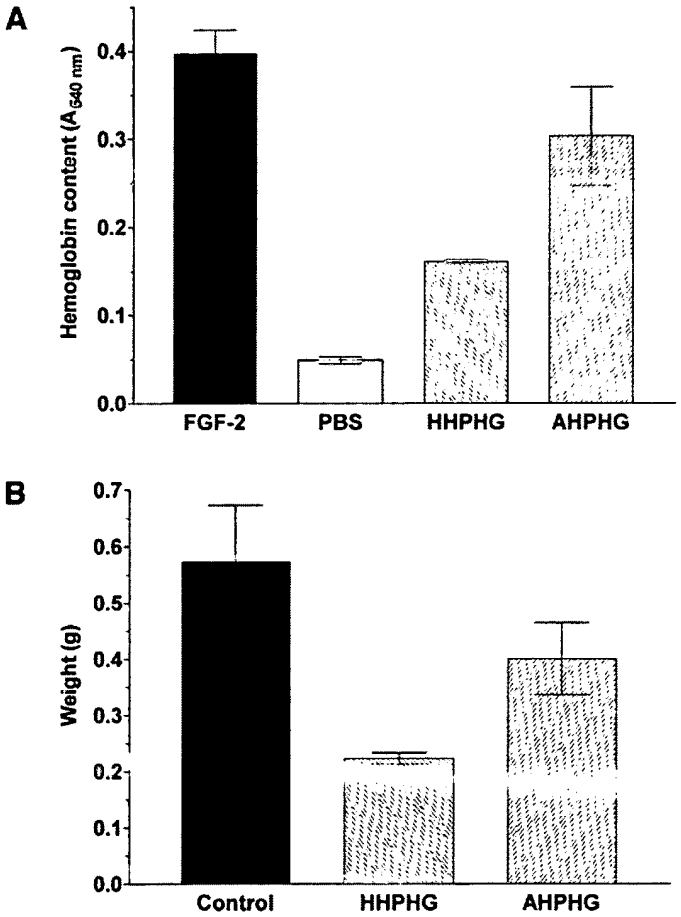

Fig. 4. The HHPHG peptide has antiangiogenic activity, which is significantly decreased in the modified AHPHG peptide. *A*, 300 μM of both peptides, HHPHG and AHPHG, were added into a mixture containing FGF-2, heparin, and Matrigel. This was then injected into the flank of a mouse following the protocol described for the Matrigel Plug assay. After 5 days in the mice, the plugs were removed and analyzed. Full (100%) angiogenesis is defined as the hemoglobin level determined by Drabkin's method in the FGF-2 containing plug minus the plug lacking FGF-2. *B*, 2×10^6 cells of the rat prostate carcinoma MatLyLu were mixed with Matrigel, together with 700 or 900 μM HHPHG and AHPHG, respectively, and then injected into the flank of a mouse following the protocol described for the modified Matrigel Plug assay. After 7 days, the animals were sacrificed; the plugs removed and weighed. All treatments were tested in triplicate and means ± SD are given.

Table 1 *Binding affinity and activity in the Matrigel plug assay of multimers of HHPHG*

Sequence	Tropomyosin binding IC_{50} (μM)	Tropomyosin binding SD	Matrigel plug % inhibition
HHPHG	92	2.8	68*
$(HHPHG)_2$	11.6	2.8	32
Ac-$(HHPHG)_2$-NH_2	26.9	2.5	41
$(HHPHG)_3$	1.25	0.31	41
Ac-$(HHPHG)_3$-NH_2	1.56	0.53	53
$(HHPHG)_4$	0.256	0.07	69
Ac-$(HHPHG)_4$-NH_2	0.245	0.12	84

NOTE. The peptides were tested in the tropomyosin binding assay as described in Fig. 1. The data are the mean and SD of $n = 3$ experiments. The peptides were also tested in the Matrigel plug assay as described in Fig. 4*A*. The data shown are from a representative experiment. HHPHG was tested at a concentration of 300 μM and the other peptides at 0.5 μM. The relationship between the number of repeats and the IC_{50} is exponential as determined by nonlinear regression analysis and is defined as $IC_{50} = 732.7 \times e^{(-2.075 \times n)}$, where n is the number of HHPHG repeats.

* Tested at 300 μM; all other peptides at 0.5 μM.

observed, providing additional support for our hypothesis that the consensus sequence, [H/P][H/P]PHG, within the H/P domain mediates these activities in HPRG. The relationship between the IC_{50} for tropomyosin and the number of repeats for the uncapped multimers is exponential as determined by nonlinear regression analysis of the data (Table 1). Similar results were obtained with the capped peptides (data not shown). The activity of Ac-$(HHPHG)_4$-NH_2 was characterized further in the Matrigel plug assay, and the titration curve generated (Fig. 5) showed an IC_{50} for Ac-$(HHPHG)_4$-NH_2 of ~600 nM, only 6-fold higher than that measured for the full-length rabbit H/P domain (1). Because Ac-$(HHPHG)_4$-NH_2 can also bind zinc, we tested for the possibility that Ac-$(HHPHG)_4$-NH_2 manifested its antiangiogenic activity by chelating zinc and inhibiting zinc-dependent processes, *e.g.*, enzymes, such as metalloproteinases, that are important for angiogenesis. To that end, Ac-$(HHPHG)_4$-NH_2 (0.5 μM) was tested in the Matrigel plug model alone or after preincubating the peptide with a 20-fold molar excess of $ZnCl_2$. If the antiangiogenic mechanism of action of Ac-$(HHPHG)_4$-NH_2 involves zinc sequestration, then saturating all possible binding zinc sites within the peptide should abrogate or decrease the antiangiogenic activity of Ac-$(HHPHG)_4$-NH_2. However, the magnitude of angiogenesis inhibition was similar regardless of whether the peptide had been preincubated with a 20-fold excess of zinc (~62 ± 22 *versus* ~60 ± 15% in the plugs where no zinc was added). $ZnCl_2$ alone, at an excess of 20-fold to the peptide concentration, did not have any significant activity in this assay (inhibition of ~5%). These results indicate that the antiangiogenic activity of Ac-$(HHPHG)_4$-NH_2 observed in the Matrigel plug model *in vivo* was most likely not due to chelation of zinc and inhibition of zinc-dependent processes.

To demonstrate that peptides derived from the H/P domain of HPRG could directly affect endothelial cells, we investigated the HUVEC binding and antiangiogenic activities of the peptide Ac-$(HHPHG)_4$-NH_2. We recently reported that biotin-rbHPRG, in the presence of zinc, binds with high affinity to FGF-2-stimulated HUVECs at two binding sites: cell surface-exposed tropomyosin (high affinity) and heparan sulfate proteoglycans (moderate affinity) (9). It was also demonstrated that the isolated H/P domain of HPRG, but not the remainder of protein (N/C fragment), can compete HPRG binding to HUVEC (9). Thus, we tested Ac-$(HHPHG)_4$-NH_2 for its ability to compete biotin-rbHPRG binding to HUVEC. Ac-$(HHPHG)_4$-NH_2, but not the N/C fragment, was able to compete the binding of biotin-rbHPRG to HUVEC *in vitro* (Fig. 6*A*) with an IC_{50} ~0.5–1.0 μM. Ac-$(HHPHG)_4$-NH_2 also inhibited FGF-2-driven HUVEC proliferation *in vitro* in a zinc-dependent manner, as has previously been demonstrated for HPRG (Ref. 1; Fig. 6*B*). In summary, Ac-$(HHPHG)_4$-

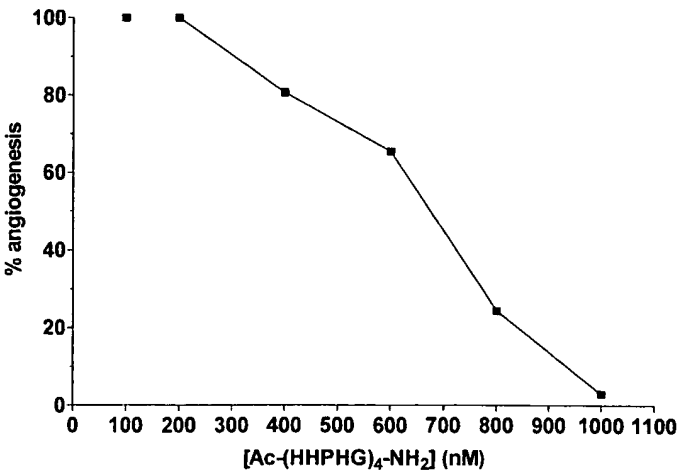

Fig. 5. The Ac-$(HHPHG)_4$-NH_2 peptide inhibits angiogenesis in the Matrigel plug assay in a dose-dependent manner. Increasing amounts of Ac-$(HHPHG)_4$-NH_2 were added directly in the Matrigel plug together with FGF-2 as described in "Materials and Methods." Full (100%) of angiogenesis is defined as the hemoglobin level determined by the Drabkin's method in the FGF-2 containing plug minus the plug lacking FGF-2.

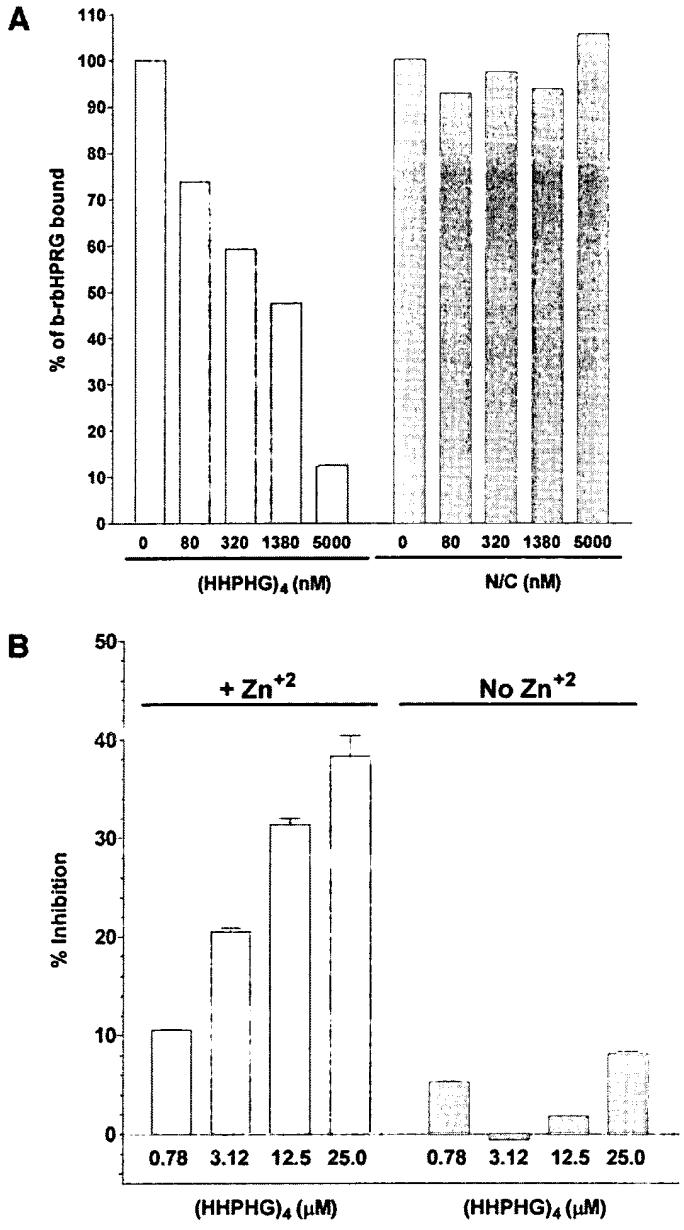

Fig. 6. The Ac-$(HHPHG)_4$-NH_2 peptide competes the binding of b-rbHPRG to HUVEC and has HUVEC-antiproliferative activity *in vitro*. *A*, the competition of 15 nM b-rbHPRG bound to FGF-2-stimulated HUVEC by increasing concentrations of $(HHPHG)_4$ peptide or the N/C fragment is shown. *B*, the effect of increasing concentrations of $(HHPHG)_4$ peptide in the presence or absence of 10 μM $ZnCl_2$ on the FGF-2-driven proliferative activity of HUVEC is shown.

NH_2 appears to bind to HUVEC and inhibits the proliferative effects of FGF-2.

Antitumor Activity of $(HHPHG)_4$. The H/P domain of rbHPRG inhibits tumor-cell-driven angiogenesis but has no direct effect on tumor cells (1). This domain was not tested in a murine model of cancer because of the difficulty in obtaining sufficient amounts of the pure H/P domain. However, because sufficient quantities of peptides could be synthesized for animal studies, we evaluated the antitumor activity of the Ac-$(HHPHG)_4$-NH_2 peptide in two fast-growing syngeneic tumor models: the 3LL and the B16F1 melanoma. The capped peptide, which has activity similar to that of uncapped $(HHPHG)_4$, was used in this study to minimize exoproteolytic degradation after injection into mice. We first determined the best route of administration at several doses of peptide using the Matrigel plug model. Ac-$(HHPHG)_4$-NH_2 was administered i.p., i.v., or s.c. into mice that had previously been implanted with Matrigel plugs containing FGF-2 and vascular endothelial growth factor (Fig. 7). The peptide demonstrated antiangiogenic activity in this model regardless of the route of administration (Fig. 7). However, angiogenesis was most effectively blocked when the peptide was administered i.v. On the basis of these preliminary results, we decided to deliver the peptide daily at two doses (25 and 50 mg/kg) by i.v. bolus Monday-Friday in the 3LL model and at a dose of 75 mg/kg using the same schedule in the B16F1 model. Treatment was initiated in both models 4 days after tumor cell inoculation. The 50 mg/kg dose in the 3LL model significantly inhibited tumor growth by 48% ($P = 0.031$) at the end of the experiment (Fig. 8*A*). Similar results were observed at the 75 mg/kg dose in the B16F1 model (Fig. 8*B*) where tumor growth was inhibited by 47% ($P = 0.053$). We could not absolutely rule out an immune response against Ac-$(HHPHG)_4$-NH_2 in these studies, which could have the effect of neutralizing the antitumor activity of the peptide. However, because of rapid progression of tumor growth in these studies, it is unlikely that the animals had sufficient time to mount a robust immune response. In addition, mouse HPRG has eight repeats of HHPHG, making generation of an immune response against this sequence less likely. In any event, significant inhibition of tumor growth was observed in both models regardless of any possible immune response demonstrating the antitumor activity of the Ac-$(HHPHG)_4$-NH_2 peptide.

DISCUSSION

In this study, we have defined the consensus region within the H/P domain of HPRG responsible for its interaction with tropomyosin and for its antiangiogenic activity. The pentapeptides from the consensus sequence of the H/P domain have moderate affinity for tropomyosin that correlates with antiangiogenic activity *in vivo*. The potency of the binding and the antiangiogenic effect increases significantly in multimers of the consensus sequence, and an exponential relationship exists between the number of consensus sequence units and affinity for tropomyosin. A tetramer of the sequence HHPHG has significant antitumor activity in two syngeneic (3LL and B16F1) models of tumor growth in mice. This correlation between affinity for tropomyosin and antiangiogenic and antitumor activities provides additional support for endothelial cell surface tropomyosin as an antiangiogenic receptor. To date, tropomyosin has been shown to be involved in binding and relaying antiangiogenic signals for HKa (5), HPRG (9),

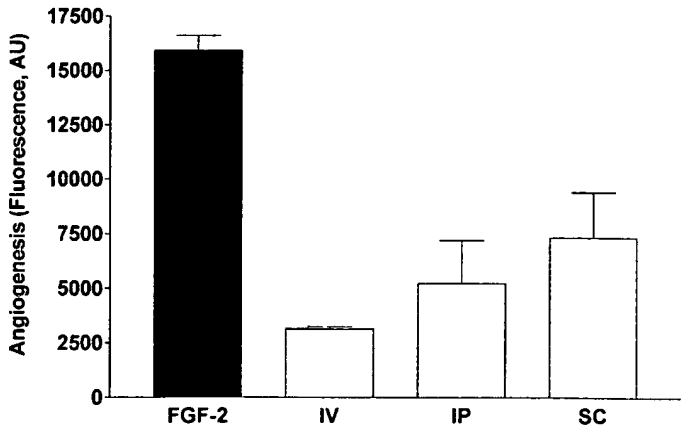

Fig. 7. The Ac-$(HHPHG)_4$-NH_2 peptide inhibits angiogenesis in the Matrigel plug assay when given i.v. Matrigel plugs containing 800 ng/ml FGF-2 and 300 ng/ml VEGF were implanted and treatment started the same day by injecting Ac-$(HHPHG)_4$-NH_2 i.v., i.p., or s.c. at 1 mg/kg/day for five days. Animals were injected with 100 μl of dextran-FITC before being sacrificed, and the plugs were analyzed. The graph shows fluorescence intensity in arbitrary units (AU).

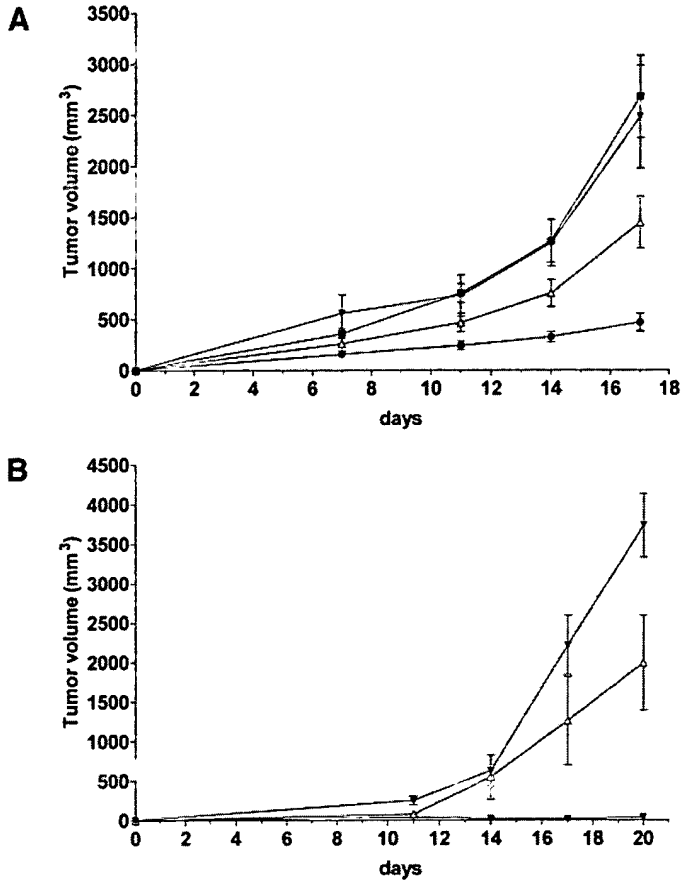

Fig. 8. The Ac-(HHPHG)$_4$-NH$_2$ peptide inhibits tumor growth in 3LL and B16F1 syngeneic models. C57Bl/6 mice were inoculated s.c. with 25 × 10^4 3LL cells or 7.5 × 10^4 B16F1 cells. After 4 days, treatment was initiated with i.v. injections of 25 (■) or 50 (△) mg/kg Ac-(HHPHG)$_4$-NH$_2$ in the 3LL model (*A*) and 75 mg/kg (△) in the B16F1 model (*B*). Treatment was administered Monday to Friday. As a negative control, mice were injected with PBS (▼); and as a positive control, animals were treated with cytoxan (●) using a metronomic regimen: 170 mg/kg, s.c., once a week. The graph shows the means of tumor volume ± SE.

and endostatin (16). Thus, tropomyosin on the surface of activated endothelial cells may be a central receptor for antiangiogenic proteins. Because tropomyosin lacks a transmembrane domain, it is likely that other proteins in the endothelial cell membrane may associate with tropomyosin and play a role in its antiangiogenic signaling.

Structurally, tropomyosin is a parallel coiled coil containing repetitions of a 7-mer motif (17). The different tropomyosin isoforms are very similar in sequence and have an acidic isoelectric point of ~4.6 (8). The H/P domain is a poly-Pro type II helix (7) in which the His residues of the repeats of the consensus sequence protrude outward. The H/P domain appears to interact with negatively charged tropomyosin only when the H/P domain acquires a positive charge either through binding to Zn^{+2} via noncharged His residues or by direct protonation of histidines induced under mild acidic conditions (9), suggesting that there is a large electrostatic contribution to the binding. This is consistent with the His residues of HPRG being essential for affinity for tropomyosin and antiangiogenic activity as demonstrated in this study. Thus, both, the H/P domain and tropomyosin are helical repetitive structures of opposite charge, suggesting that the overall affinity may be the sum of several coordinated individual interactions involving these repetitive units. Interestingly, a basic pentapeptide from the β chain of hepatocyte growth factor (HHRGK) has also been shown to have antiangiogenic activity (18). This peptide has sequence similarity and basic character analogous to HHPHG. In light of the electrostatic nature of the interaction of the H/P domain and derived peptides with tropomyosin, it is tempting to speculate that HHRGK and other small antiangiogenic basic peptides (19) may be acting through binding to tropomyosin on the surface of endothelial cells.

The affinity of the peptides examined here for tropomyosin increases exponentially with the number of repeats of HHPGH, A corollary of this relationship is that a peptide comprised of 5 HHPGH units would have a predicted IC_{50} of 23 nM in the tropomyosin binding assay (using the equation derived in Table 1). This value is practically identical to that of the full H/P domain of HPRG (IC_{50} when competing with 10 nM HPRG ~14 nM). Therefore, it is possible that the affinity of the H/P domain for tropomyosin, and conceivably its full antiangiogenic activity, would be recapitulated by a pentamer of HHPHG units, nearly a 4-fold reduction in size from the H/P domain. The consequences of this possibility for understanding the interaction of the H/P domain with tropomyosin and for the development of an antiangiogenic agent are currently under investigation.

In summary, we have characterized some of the biochemical and biophysical aspects of the interaction of HPRG with tropomyosin that mediate the antiangiogenic activity of this relatively abundant plasma protein and have identified a 16-mer peptide with significant antiangiogenic and antitumor activity derived from a consensus sequence within the H/P domain of HPRG.

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